Remarks

Reconsideration and withdrawal of the rejections of the claims, in view of the amendments and remarks herein, is respectfully requested. Claims 1, 47, 67, 74, and 78 are amended, and claims 83-94 are added. The amendments are intended to advance the application and are not intended to concede to the correctness of the Examiner's position or to prejudice the claims prior to amendment, which claims are present in a continuation of the above-referenced application. Claims 1, 3-6, 9, 11-12, 15, 18, 20-21, 24-39, 41-45, 47, 60, 64, 67, 69-71, 74, 76-78, and 80-94 are pending.

The Examiner is thanked for the courtesies extended to Applicant's Representative in the telephonic interview conducted on March 11, 2005, in which the Amendment filed on December 13, 2004 was discussed.

The 35 U.S.C. § 112, Second Paragraph, Rejections

The Examiner rejected claims 1, 3-6, 9, 11-12, 15, 18, 20-21, 24-39, 41-45, 47, 60, 67, 69-71, 74, 76-78, and 80-82 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. In particular, the Examiner asserts that the phrase "a reduced number of a combination of transcription factor binding sequences, intron splice sites, poly(A) addition sites and/or promoter sequences" is indefinite. This rejection is respectfully traversed.

It is Applicant's position that the phrases "transcription factor binding sequences," "intron splice sites," "poly(A) addition sites" and "promoter sequences" are conventionally used and understood by the art. See, e.g., U.S. Patent No. 5,670,356 ("transcription factor binding sites"), WO 97/47358 ("intron splice sites"), Iannaconne et al., Plant Mol. Biol., 34:485 (1997) ("polyA sequences"), and Pan et al., Nucl. Acids Res., 27:1094 (1999) ("prokaryotic promoters," "poly(A) signals," and "exon-intron boundaries"), all cited against the present application under 35 U.S.C. § 103(a), and Faisst and Meyer, Nucl. Acids Res., 20:3 (1992), cited at page 50, lines 6-7 of Applicant's specification), which disclose a compilation of vertebrate encoded transcription factors.

The Examiner also rejected claims 1, 3-6, 9, 11-12, 15, 18, 20-21, 24-39, 41-45, 47, 60, 67, 69-71, 74, 76-78, and 80-82 under 35 U.S.C. § 112, second paragraph, as it is purportedly unclear whether any non-preferred codons may be present in the second synthetic nucleic acid and how the second synthetic nucleic acid differs from the wild-type sequence. This rejection is respectfully traversed.

Claims 1, 47, 67, 74 and 78 recite that the codons in the second synthetic nucleic acid molecule (or polynucleotide) that are different than the codons in the wild-type (or parent) nucleic acid sequence are mammalian high usage codons selected to result in the second synthetic nucleic acid molecule (or polynucleotide) having a reduced number of a combination of regulatory sites relative to the wild-type (or parent) nucleic acid sequence, wherein the codons which differ in the first synthetic nucleic acid molecule (or polynucleotide) relative to the second synthetic nucleic acid molecule (or polynucleotide) are mammalian codons selected to result in the first synthetic nucleic acid molecule (or polynucleotide) having a reduced number of regulatory sites (emphasis added). Moreover, Applicant's specification discloses that mammalian high usage codons are selected to replace codons in a wild-type (or parent) nucleic acid molecule that include an undesirable regulatory site, and that to remove regulatory sites introduced by codon replacement, other mammalian codons may be employed (see page 35, lines 24-29, page 37, lines 17-24, page 46, lines 3-28, and page 51, lines 5-10).

Thus, it is clear that codons other than the most frequently used mammalian codons may be present in the second synthetic nucleic acid molecule (or polynucleotide). Moreover, with respect to the differences between the second synthetic nucleic acid molecule (or polynucleotide) and the wild-type (or parent) nucleic acid molecule, it is clear that the second synthetic nucleic acid sequence (or polynucleotide) has an increased number of mammalian codons and a reduced number of a combination of regulatory sites, e.g., mammalian transcription factor binding sequences, intron splice sites, poly(A) addition sites and/or promoter sequences, relative to a wild-type (or parent) nucleic acid sequence.

Accordingly, withdrawal of the 35 U.S.C. § 112, second paragraph, rejections is respectfully requested.

The 35 U.S.C. § 112, First Paragraph, Rejections

The Examiner rejected claims 1, 3-6, 9, 11-12, 15, 20-21, 24-33, 35-39, 41-45, 47, 60, 67, 69-70, and 81-82 under 35 U.S.C. § 112, first paragraph. The Examiner asserts that, although the specification is enabling for 1) a variant of a parent DNA molecule encoding a reporter polypeptide identical to a reporter polypeptide encoded by the parent DNA, having more than 25% of the codons altered and having a reduced number of transcription regulatory sequences than a mammalian codon optimized variant of the parent nucleic acid, 2) a variant of a parent DNA molecule encoding a luciferase having 90% identity to the polypeptide encoded by SEQ ID NO:2 and having more than 25% of the codons altered and having a reduced number of transcription regulatory sequences than a mammalian codon optimized variant of SEQ ID NO:2, or 3) to any nucleic acid which will hybridize to SEQ ID NO:9 under high stringency conditions and encode a polypeptide having luciferase activity, it does not reasonably provide enablement for any variant DNA molecule encoding a reporter polypeptide having at least 90% identity to a wild-type reporter polypeptide, having more than 25% of the codons altered and having a reduced number of transcription regulatory sequences than a mammalian codon optimized version of the parent nucleic acid or to any nucleic acid which will hybridize to SEQ ID NO:9 under medium stringency conditions. This rejection, as it may be maintained with respect to the pending claims, is respectfully traversed.

First, it is unclear to Applicant from the Office Action how medium stringency hybridization conditions are not enabled but high stringency conditions are enabled, as Applicant's specification discloses both (see page 20, lines 14-27). Further clarification on this issue is respectfully requested.

With respect to reporter polypeptides, such as GFP, beetle luciferase, GUS, and CAT, as well as beta-lactamase (see, e.g., the abstracts for Stapleton et al., Antimicrob. Agents Chemother., 43:1881 (1999); Bouthors et al., Protein Eng., 12:313 (1999); Sirot et al., Antimicrob. Agents Chemother., 41:1322 (1997); and Voladri et al., J. Bacteriol., 178:7248 (1996), a copy of each is enclosed herewith), Applicant has provided evidence that it is well within the skill of the art to introduce substitutions into various reporter proteins and yield a variant protein with the activity of the corresponding wild-type reporter protein.

Therefore, withdrawal of the § 112(1) rejection is appropriate and is respectfully requested.

The 35 U.S.C. § 103(a) Rejection

The Examiner rejected claims 1, 3-6, 9, 11-12, 15, 20-21, 24-39, 41-45, 47, 60, 67-70, 74, 76-77, and 81-82 under 35 U.S.C. § 103(a) as being unpatentable over Sherf et al. (U.S. Patent No. 5,670,356) in view of Zolotukhin et al. (U.S. Patent No. 5,874,304), Donnelly et al. (WO 97/47358), Iannacone et al. (Plant Mol. Biol., 34:485 (1997)) and Pan et al. (Nucl. Acids Res., 27:1094 (1999)). As this rejection may be maintained with respect to the pending claims, it is respectfully traversed.

Sherf et al. disclose a synthetic firefly luciferase gene (*luc*$^+$) in which 3 internal palindromic sequences, 5 restriction endonuclease sites, 4 glycosylation sites, and 6 transcription factor binding sites that were present in the unmodified sequence were removed, and codons were altered at sequences specified in Table 2 to codons preferred ("more common") in mammalian cells, relative to a wild type firefly luciferase gene (*luc*). Of the twenty 6 to 30 bp regions which were modified, 6 regions included modifications with a dual purpose, i.e., one region was modified to eliminate a glycosylation site and a transcription factor binding site that was present in the unmodified sequence, three regions were modified to eliminate a transcription factor binding site that was present in the unmodified sequence and improve codon usage, one region was modified to eliminate two transcription factor binding sites (but not improve codon usage) that were present in the unmodified sequence, and another region was modified to improve codon usage and eliminate a restriction endonuclease recognition site.

Sherf et al. also disclose that a vector encoding Luc$^+$ or Luc was introduced to four mammalian cell lines. NIH3T3 and HeLa cells transfected with *luc*$^+$ DNA had significantly higher levels of luciferase activity relative to NIH3T3 and HeLa cells transfected with *luc* DNA (Table 3), while CHO and CV-1 cells transfected with *luc*$^+$ or *luc* DNA had comparable luciferase activity. However, it is unclear what alterations in *luc*$^+$ DNA increased luciferase activity in mammalian cells, and why those alterations did not uniformly increase luciferase activity in all the tested mammalian cells. In contrast, a synthetic *Renilla* luciferase gene of the

invention was expressed at significantly higher levels relative to a wild type *Renilla* luciferase gene in NIH3T3, HeLa, CHO and CV-1 cells (Table 10).

Sherf et al. do not teach or suggest that modification of a parent sequence to remove palindromic sequences, restriction endonuclease sites, glycosylation sites, and transcription factor binding sites may introduce other undesirable sequences. Nor do Sherf et al. disclose or suggest replacing at least 25% of the codons in a parent sequence with selected mammalian codons, thereby reducing a large number of transcription factor binding sequences in the parent sequence.

A humanized version of a green fluorescent protein (GFP) gene is disclosed in Zolotukhin et al. in which 88/238 of the codons in the gene were altered (column 13, lines 1-4). Zolotukhin et al. do not disclose or suggest that codon optimization of a parent sequence may introduce undesirable sequences.

WO 97/47358 discloses the preparation of synthetic hepatitis C virus (HCV) genes. In particular, it is disclosed that codons in the corresponding wild-type gene that are not the most commonly employed in humans, are replaced with an optimal codon. If a CG is created by that codon replacement, i.e., the third nucleotide in the replaced codon is C and the first nucleotide in the adjacent codon is G, WO 97/47358 discloses that a different codon is selected based on Table 5 in Lathe et al. (J. Mol. Biol., 183:1 (1985)) (page 17). Once all codon replacements are made, it is disclosed that the codon optimized gene is inspected for undesired sequences such as ATTTA sequences, inadvertent creation of intron splice sites, and unwanted restriction enzyme sites, which are then eliminated by substituting codons (pages 17-18). The bias away from CG residues during codon optimization would reduce overall CG content in the final synthetic sequence unless codon substitution to remove undesired sequences resulted in an increase in CG dinucleotides in adjacent codons (thus defeating the reasoning behind avoiding CGs in adjacent codons). In that regard, note that the synthetic click beetle and *Renilla* luciferase genes described in the Examples had increased CG content relative to the respective parent sequence.

WO 97/47358 provides no details of the sequence of any undesirable sites including intron splice sites which are to be eliminated or how to substitute codons to remove ATTTA sequences, polyA sequences, splicing sites and A or T strings > 4. Further, there is no

recognition in WO 97/47358 that codon optimization may introduce transcription factor binding sequences or that transcription factor binding sequences may be removed from sequences.

Iannacone et al. disclose synthetic *Bacillus thuringienesis* Bt43 genes (abstract) which encode an insect toxin. To prepare those genes, Iannacone et al. modified the nucleotide sequence of Bt43 in four target regions to avoid sequences which might destabilize mRNA, sequences such as ATTTA sequences, polyA sequences, splicing sites and A or T strings > 4, and to improve codon usage for plant expression (abstract and page 490). However, splicing sites were apparently not removed (Table 1). No details are provided in Iannaconne et al. on what polyA sequences were identified and how codons were selected to replace those identified sites. Moreover, Iannaconne et al. did not seek to eliminate transcription factor binding sequences.

Five constructs, one with the wild type Bt43 gene and four with synthetic Bt43 genes, i.e., BtE, BtF, BtH and BtI (see Figure 2 and Table 1), were introduced to eggplant or *Solanum integrifolium* cultures, and transgenic plants regenerated. Bt43-specific polyA+ RNA in the plants was detected by Northern blot analysis (Figure 4).

It is disclosed in Iannacone et al. that no Bt43-specific bands were detected in lanes for plants with the wild type Bt43 gene even after long exposures (page 491). Interestingly, plants expressing the BtE gene had higher levels of Bt43-specific polyA+ mRNA than plants expressing the BtF gene, a gene which had fewer $(A)_{>4}$ and $(T)_{>4}$ strings, one less ATTTA sequence, and more codons modified relative to the BtE gene. In fact, no full size Bt43-specific mRNA was detected in BtF transgenic plants in contrast to BtE transgenic plants (page 494). Thus, the additional modifications in BtF relative to BtE, i.e., additional codon substitutions, and a reduced number of ATTTA sequences and A or T strings > 4, reduced full length mRNA.

And although full length BtE polyA+ RNA was present in BtE transgenic plants, no Bt toxin was produced, leading the authors to conclude that a 1.2 Kb unmodified domain in the BtE gene is a major candidate for translational blockade (page 494).

The authors of Iannacone et al. conclude that the increased level of Bt43-specific mRNA in BtE and BtF transgenic plants compared to wild type Bt43 transgenic plants could be related to the elimination of destabilizing sequences and that the AUUUA string in wild type Bt43 is a major candidate for the instability and untranslatability of Bt43 mRNA (page 494).

There is no disclosure or suggestion in Iannacone et al. that the alteration of the sequence of a gene such as a reporter gene to remove sequences such as ATTTA sequences, polyA sequences, splicing sites and A or T strings > 4, and to improve codon usage for plant expression may introduce undesirable sequences.

Pan et al. describe a synthetic gene derived from the merozoite surface protein-1 gene (*msp*-1) of *Plasmodium falciparum*. The synthetic gene was prepared by first back translating the corresponding wild type gene using random (not preferred) human codon replacement, choosing one master sequence, and then modifying the master synthetic sequence via alternate codon replacement to eliminate sequences that might be detrimental to efficient transcription and translation, i.e., endonuclease cleavage sites, prokaryotic promoters, poly(A) signals, exon-intron boundaries, prokaryotic factor-independent RNA polymerase terminators, inverted repeats, and long runs of purines (page 1095). Notably Pan et al. did not seek to eliminate transcription factor binding sequences in *msp*-1 and did not recognize that codon optimization may introduce transcription factor binding sequences. Nor does Pan et al. disclose the sequences for prokaryotic promoters, poly(A) signals, or exon-intron boundaries that were to be identified for removal.

In order for the Examiner to establish a *prima facie* case of obviousness, three base criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on Applicant's disclosure. M.P.E.P. § 2142 (citing In re Vaeck, 947 F.2d 488, 20 U.S.P.Q.2d (BNA) 1438 (Fed. Cir. 1991)).

The combination of references does not disclose or suggest Applicant's invention as each reference discloses a different way to modify the coding sequence of a different gene, i.e., viral genes, a gene from a parasite associated with malaria, an insect toxin gene, or a reporter gene, to increase expression, i.e., Zolotukhin et al. disclose codon modification alone to codons employed more frequently in one organism generally throughout a green fluorescent protein gene, Sherf et

al. disclose limited and targeted modification (modifications in 20 regions of 6 to 30 bp) of a firefly luciferase sequence to introduce or remove cloning sites, alter insect codons to mammalian codons, and to remove post-translation modification sites, secondary structure, and transcription factor binding sites, Iannacone et al. disclose targeted modification of four regions of a toxin gene to alter *Bacillus* codons to plant codons, and to remove polyA sequences, ATTTA sequences and strings of A or T > 4, WO 97/47358 describes codon replacement to more commonly employed codons combined with further codon substitution to remove CG residue in adjacent codons, and then inspection for ATTTA sequences, intron splice sites, and unwanted restriction enzyme sites, and Pan et al. disclose random human codon replacement yielding a population of synthetic sequences with codon substitutions, choosing one master synthetic sequence, and then modifying the master synthetic sequence via alternate codon replacement to eliminate sequences that might be detrimental to efficient transcription and translation, i.e., endonuclease cleavage sites, prokaryotic promoters, poly(A) signals, exon-intron boundaries, prokaryotic factor-independent RNA polymerase terminators, inverted repeats, and long runs of purines.

Thus, while there is a general teaching in the combination of cited documents to alter codons and/or remove certain undesired sequences in a selected sequence, none of the cited documents teaches or suggests that codon alterations, to prepare a sequence with codons employed more frequently in an evolutionarily divergent organism optionally in conjunction with removal of restriction enzyme sites, ATTTA sequences, splice sites, polyA sites, A or T strings, CG dinucleotides in adjacent codons, prokaryotic promoters, inverted repeats and prokaryotic factor-independent RNA polymerase terminators, may create transcription factor binding sites. Moreover, none of the cited documents discloses or suggests removal of transcription factor binding sites from a codon optimized gene.

And although one of skill in the art in possession of the cited documents may be motivated to alter the codons of a particular sequence, there is no direction in the combination of cited documents which yields Applicant's invention. It is only with hindsight, i.e., with knowledge of Applicant's invention, that one of skill in the art, picking and choosing from the cited documents, is directed to Applicant's invention. That is, to arrive at Applicant's invention, one of skill in the art in possession of the cited art, would chose to modify a reporter gene (Sherf

et al., Zolotukhin et al. and possibly Iannaconne et al.), rather than a non-reporter gene (WO 97/47358 and Pan et al.), by codon replacement of an entire opening reading frame (Zoltukhin et al., WO 97/47358, and Pan et al.) rather than a portion of an open reading frame (Sherf et al. and Iannaconne et al.), with subsequent additional alterations (WO 97/47358 and Pan et al.) rather than without further alterations (Zolotukhin et al.) or concurrently with other alterations (Sherf et al. and likely Iannaconne et al.), where codons are replaced with preferred mammalian codons (Sherf et al., Zolotukhin et al., WO 97/47358, and Iannaconne et al.), rather than random codon replacement (Pan et al.).

Moreover, to arrive at Applicant's invention, one of skill in the art in possession of the cited documents would choose to identity transcription factor binding sites (Sherf et al.), promoter sequences (Pan et al.), splice sites (WO 97/47358, Iannaconne et al., and Pan et al.), and polyA sites (Iannaconne et al. and Pan et al.), as sequences that may be removed by codon replacement although Sherf et al. teach removal of internal palindromic sequences, restriction endonuclease sites, glycosylation sites, and transcription factor binding sites, WO 97/47358 disclose removing ATTTA sequences, inadvertent creation of intron splice sites, and unwanted restriction enzyme sites, Iannaconne et al. disclose removing ATTTA sequences, polyA sequences, splicing sites and A or T strings > 4, Pan et al. disclose removing endonuclease cleavage sites, prokaryotic promoters, poly(A) signals, exon-intron boundaries, prokaryotic factor-independent RNA polymerase terminators, inverted repeats, and long runs of purines, and Zolotukhin et al. do not mention removal of any regulatory sequence in a coding region.

Further, none of the cited documents discloses or suggests the use of software to identify particular regulatory sites, such as mammalian transcription factor binding sequences in a database of transcription factor binding sequences.

The Examiner is requested to consider that after codon optimization in conjunction with removal of non-transcription factor binding sites in click beetle and *Renilla* luciferase nucleotide sequences, Applicant identified about 100 and about 60 transcription factor binding sequences, respectively. Further codon replacement to remove those sequences yielded synthetic click beetle and *Renilla* luciferase sequences with 50 and 20 new transcription factor binding sites, respectively, i.e., they were introduced by codon replacement (Examples 1 and 3). The vast

majority of the introduced sequences were subsequently removed to yield a synthetic nucleic acid molecule of the invention.

With regard to the alleged motivation provided by the cited documents, if altering codon composition in an open reading frame to codons preferred in a heterologous host alone increases expression in the heterologous host, then there would be no motivation for the art worker to make any other changes, e.g., those which may reduce aberrant transcription.

Further, one of ordinary skill in the art in possession of the cited art would have no reasonable expectation that any particular set of changes may improve activity in a gene that is to be expressed in a highly evolutionarily distinct cell. For example, codon alterations and a reduction in polyA sequences, strings of A or T > 4 and ATTTA sequences in a Bt43 sequence yielded mRNA but did not yield a detectable protein (BtE in Iannacone et al.), and further codon alterations and a further reduction in strings of A or T > 4 and ATTTA sequences did not yield detectable full length mRNA (BtF in Iannacone et al.). Thus, an increase in codon substitutions and a decrease in RNA destabilization sequences in a synthetic gene do not necessarily improve the transcriptional characteristics of the synthetic gene relative to the reference gene. In addition, it is unclear what changes to the Bt43 (Iannacone et al.), HCV genes (WO 97/47358), *msp*-1 gene (Pan et al.) or *luc* (Sherf et al.) sequence result in improved activity in a heterologous host and why replacement of codons in *luc* with codons preferred in mammals and other alterations which resulted in luc^+ did not improve luciferase activity in all mammalian cells which expressed Luc^+.

Accordingly, withdrawal of the § 103 rejection is respectfully requested.

CONCLUSION

Applicant respectfully submits that the claims are in condition for allowance and notification to that effect is earnestly requested. The Examiner is invited to telephone Applicant's attorney (612) 373-6959 to facilitate prosecution of this application.

If necessary, please charge any additional fees or credit overpayment to Deposit Account No. 19-0743.

Respectfully submitted,

KEITH V. WOOD ET AL.

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Date September 22, 2005 By _____
Janet E. Embretson
Reg. No. 39,665

CERTIFICATE UNDER 37 CFR 1.8: The undersigned hereby certifies that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail, in an envelope addressed to: Mail Stop RCE, Commissioner of Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on this 22 day of September, 2005.

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Dawn M. Rose
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1: Antimicrob Agents Chemother. 1999 Aug;43(8):1881-7. Related Articles, Links

Construction and characterization of mutants of the TEM-1 beta-lactamase containing amino acid substitutions associated with both extended-spectrum resistance and resistance to beta-lactamase inhibitors.

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Extended-spectrum TEM beta-lactamases (ESBLs) do not usually confer resistance to beta-lactamase inhibitors such as clavulanate or tazobactam. To investigate the compatibility of the two phenotypes we used site-directed mutagenesis of the bla(TEM-1) gene to introduce into the TEM-1 beta-lactamase amino acid substitutions that confer the ESBL phenotype: TEM-12 (Arg164-->Ser), TEM-26 (Arg164-->Ser plus Glu104-->Lys), TEM-19 (Gly238-->Ser), and TEM-15 (Gly238-->Ser plus Glu104-->Lys). These were combined with three sets of substitutions that confer inhibitor resistance: TEM-31 (Arg244-->Cys), TEM-33 (Met69-->Leu), and TEM-35 (Met69-->Leu and Asn276-->Asp). Introduction of the Arg244-->Cys substitution gave rise to inhibitor-resistant hybrid enzymes that either lost ESBL activity (TEM-12, TEM-15, and TEM-19) or had reduced activity (TEM-26) against ceftazidime. In contrast, the introduction of Met69-->Leu or Met69-->Leu plus Asn276-->Asp substitutions did not significantly affect the abilities of the enzymes to confer resistance to ceftazidime, although increased susceptibility to cefotaxime was observed with Escherichia coli strains that expressed the TEM-19 and TEM-26 beta-lactamases. With the exception of the TEM-12 beta-lactamase, introduction of the Met69-->Leu substitution did not give rise to enzymes with increased resistance to clavulanate compared to that of the TEM-1 beta-lactamase. However, introduction of the double substitution Met69-->Leu plus Asn276-->Asp in the ESBLs did give rise to low-level (TEM-19, TEM-15, and TEM-26) or moderate-level (TEM-12) clavulanate resistance. None of the hybrid

enzymes were as resistant to clavulanate as the corresponding inhibitor-resistant TEM beta-lactamase mutant, suggesting that active-site configuration in the ESBLs limits the degree of clavulanate resistance conferred.

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1: Protein Eng. 1999 Apr;12(4):313-8.

Related Articles, Links

Site-directed mutagenesis of residues 164, 170, 171, 179, 220, 237 and 242 in PER-1 beta-lactamase hydrolysing expanded-spectrum cephalosporins.

Bouthors AT, Delettre J, Mugnier P, Jarlier V, Sougakoff W.

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The class A beta-lactamase PER-1, which displays 26% identity with the TEM-type extended-spectrum beta-lactamases (ESBLs), is characterized by a substrate profile similar to that conferred by these latter enzymes. The role of residues Ala164, His170, Ala171, Asn179, Arg220, Thr237 and Lys242, found in PER-1, was assessed by site-directed mutagenesis. Replacement of Ala164 by Arg yielded an enzyme with no detectable beta-lactamase activity. Two other mutants, N179D and A164R+N179D, were also inactive. Conversely, a mutant with the A171E substitution displayed a substrate profile very similar to that of the wild-type enzyme. Moreover, the replacement of Ala171 by Glu in the A164R enzyme yielded a double mutant which was active, suggesting that Glu171 could compensate for the deleterious effect of Arg164 in the A164R+A171E enzyme. A specific increase in kcat for cefotaxime was observed with H170N, whereas R220L and T237A displayed a specific decrease in activity towards the same drug and a general increase in affinity towards cephalosporins. Finally, the K242E mutant displayed a kinetic behaviour very similar to that of PER-1. Based on three-dimensional models generated by homology modelling and molecular dynamics, these results suggest novel structure-activity relationships in PER-1, when compared with those previously described for the TEM-type ESBLs.

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1: Antimicrob Agents Chemother. 1997 Jun;41(6):1322-5. Related Articles, Links

A complex mutant of TEM-1 beta-lactamase with mutations encountered in both IRT-4 and extended-spectrum TEM-15, produced by an Escherichia coli clinical isolate.

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Escherichia coli GR102 was isolated from feces of a leukemic patient. It expressed different levels of resistance to amoxicillin or ticarcillin plus clavulanate and to the various cephalosporins tested. The double-disk synergy test was weakly positive. Production of a beta-lactamase with a pI of 5.6 was transferred to E. coli HB101 by conjugation. The nucleotide sequence was determined by direct sequencing of the amplification products obtained by PCR performed with TEM gene primers. This enzyme differed from TEM-1 (blaT-1B gene) by four amino acid substitutions: Met-->Leu-69, Glu-->Lys-104, Gly-->Ser-238 and Asn-->Asp-276. The amino acid susbstitutions Leu-69 and Asp-276 are known to be responsible for inhibitor resistance of the IRT-4 mutant, as are Lys-104 and Ser-238 substitutions for hydrolytic activity of the extended-spectrum beta-lactamases TEM-15, TEM-4, and TEM-3. These combined mutations led to a mutant enzyme which conferred a level of resistance to coamoxiclav (MIC, 64 microg/ml) much lower than that conferred by IRT-4 (MIC, 2,048 microg/ml) but higher than that conferred by TEM-15 or TEM-1 (MIC, 16 microg/ml). In addition, the MIC of ceftazidime for E. coli transconjugant GR202 (1 microg/ml) was lower than that for E. coli TEM-15 (16 microg/ml) and higher than that for E. coli IRT-4 or TEM-1 (0.06 microg/ml). The MICs observed for this TEM-type enzyme were related to the kinetic constants Km and k(cat) and the 50% inhibitory concentration, which were intermediate between those observed for IRT-4 and TEM-15. In conclusion, this new type of complex mutant derived from TEM-1 (CMT-1) is able to confer resistance at a very low level to inhibitors and at a low level to

extended-spectrum cephalosporins. CMT-1 received the designation TEM-50.

PMID: 9174192 [PubMed - indexed for MEDLINE]

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1: J Bacteriol. 1996 Dec;178(24):7248-53. Related Articles, Links

Structure-function relationships among wild-type variants of Staphylococcus aureus beta-lactamase: importance of amino acids 128 and 216.

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beta-Lactamases inactivate penicillin and cephalosporin antibiotics by hydrolysis of the beta-lactam ring and are an important mechanism of resistance for many bacterial pathogens. Four wild-type variants of Staphylococcus aureus beta-lactamase, designated A, B, C, and D, have been identified. Although distinguishable kinetically, they differ in primary structure by only a few amino acids. Using the reported sequences of the A, C, and D enzymes along with crystallographic data about the structure of the type A enzyme to identify amino acid differences located close to the active site, we hypothesized that these differences might explain the kinetic heterogeneity of the wild-type beta-lactamases. To test this hypothesis, genes encoding the type A, C, and D beta-lactamases were modified by site-directed mutagenesis, yielding mutant enzymes with single amino acid substitutions. The substitution of asparagine for serine at residue 216 of type A beta-lactamase resulted in a kinetic profile indistinguishable from that of type C beta-lactamase, whereas the substitution of serine for asparagine at the same site in the type C enzyme produced a kinetic type A mutant. Similar bidirectional substitutions identified the threonine-to-alanine difference at residue 128 as being responsible for the kinetic differences between the type A and D enzymes. Neither residue 216 nor 128 has previously been shown to be kinetically important among serine-active-site beta-lactamases.

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